Multitarget Affinity/Specificity Screening of Natural Products: Finding and Characterizing High-Affinity Ligands from Complex Mixtures by Using High-Performance Mass Spectrometry

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In this work we describe a high-throughput screening approach based on electrospray ionization−Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR) that rapidly interrogates the noncovalent interaction between RNA-based drug targets and components derived from a bacterial natural product library. The screening process detects molecules present in the natural product library that bind to a synthetic RNA target that mimics the prokaryotic 16S rRNA A-site, while simultaneously measuring specificity for the synthetic A-site target using a control RNA target that lacks the critical structural element of the A-site construct. This screening approach known as multitarget affinity/specificity screening (MASS) demonstrated the expected binding of paromomycin from a fractionated natural product library derived from *Streptomyces rimosus* sp. *paromomycinus*. A new molecule was observed to bind with specificity to the 16S A-site RNA construct. MS/MS characterization of this species yielded partial structural information suggesting it is an aminoglycoside consisting of a paromomycin core with one or more modified rings. This work demonstrates the tremendous utility of MASS for screening natural product fractions against macromolecular targets.

The characterization of biologically active fractions from collections of natural products presents many challenges. The many issues include detection of active compounds present at low concentrations in a background of other active species and "false" positives resulting from the summed activity of many weakly active compounds. Historically, mixtures of similar compounds are separated using chromatographic methods prior to screening.

In this paper we demonstrate that electrospray ionization mass spectrometry (ESI-MS) can be used as a rapid screening method for identification of active compounds from crude mixtures. ESI-MS allows the simultaneous analysis of mixtures of compounds based on their unique molecular masses. In addition, active compounds can be identified directly from their noncovalent complexes with target molecules. Control targets can be included in the screening mixture to provide a measure of binding specificity. ESI-MS has high sensitivity and resolving power that facilitates the analysis of trace levels of complex mixtures; such analyses can be implemented in a high-throughput modality with the appropriate robotic interfaces.

At the core of this approach is the use of electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FTICR) mass spectrometry (MS)[1] to characterize noncovalent complexes comprised of a molecular target (e.g., structured RNA or protein) and a small molecule ligand. Mass measurements of the intact complex, exact mass measurements of the affinity-selected ligand, and subsequent tandem MS measurements are used to gain insight into the composition and structure of the binding species.

There is a growing body of literature that demonstrates that electrospray ionization mass spectrometry (ESI-MS) can be used to study the gas-phase properties of ions from noncovalent biomolecular complexes.[2−5] These include protein−ligand, protein−protein, protein−DNA, DNA−DNA, RNA−protein, DNA−ligand, and RNA−ligand complexes.[6] Molecular interactions with dissociation constants ranging from nM to mM can be characterized using ESI-MS.[7] We, and others, have shown that ESI-MS can be used as a quantitative tool to measure molecular dissociation constants that agree with solution measurements.[8−11] These benefits make ESI-MS a powerful screening technique for identification of novel compounds that bind to a macromolecular target of interest.

Targeting structured RNA presents new opportunities for drug discovery. Structured RNA plays multiple, essential roles in protein production. In addition to the obvious role of mRNA carrying the linear coded message for translation into proteins, structured regions of certain mRNAs control the level of protein production by binding to proteins, and binding of small molecules to these structures may actually increase protein production. Many viral and a number of cellular mRNAs contain a structured 5′-untranslated region that may be of interest as a drug target. This region, known as the internal ribosome entry site (IRES), enables binding to a ribosome and initiation of protein translation without the presence of a traditional 5′ cap. One of the most studied and important structured RNA targets is the prokaryotic ribosomal RNA. The aminoglycoside class of antibiotics causes misreading of the genetic code by binding to the 16S RNA subunit of the prokaryotic ribosome. Binding occurs in a structured region of the 16S RNA known as the A-site.

We have recently employed ESI-FTICR to measure binding constants between aminoglycoside antibiotics and the decoding region of the prokaryotic 16S rRNA (the A-site).[11] In addition, we have shown that it is possible to detect specific interactions between two closely related model RNA constructs corresponding to the decoding sites of the prokaryotic and eukaryotic rRNA and a collection of aminoglycoside antibiotics.[12]

Subsequently, we demonstrated that by multiplexing both targets and compound collections it is possible to

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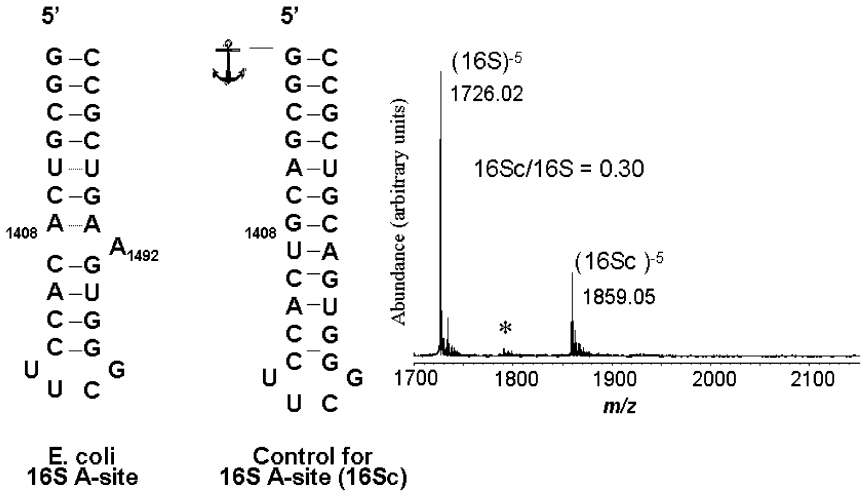

Figure 1. Sequence and proposed secondary structure for *E. coli* 16S A-site (16S) and control 16S A-site (16Sc) synthetic constructs. Base numbering is in reference to full length *E. coli* 16S RNA. Anchor icon on 16SC refers to the neutral mass tag (see Experimental Section). Right panel shows a typical mass spectrum of 16S and 16SC. The peak *m/z* values represent the monoisotopic species. The asterisk denotes the presence of a synthetic impurity, 16SC RNA without the neutral mass tag.

evaluate the affinity of more than 67 000 putative ligand−substrate pairs in a 24 h screening run.[13] This scheme, termed multitarget affinity/specificity screening (MASS), exemplifies the utility of high-performance mass spectrometry for rapid determination of binding properties in a massively parallel fashion and represents a novel paradigm for drug discovery using multiple macromolecular targets against a collection of small molecule ligands. In this work we describe the extension of the MASS paradigm to the high-throughput screening of crude natural products. The utility as a drug discovery tool is demonstrated with a model system comprised of bacterial broth extracts derived from cultures of *Streptomyces* sp. (which are known to produce aminoglycoside antibiotics) screened against the 16S rRNA A-site target described previously.[12,14,15]

Experimental Section

Bacterial Strain and Culture Conditions. A dried sample of American Type Culture Collection 14827 (ATCC14827), *Streptomyces rimosus* sp. *paromomycinus*, was dissolved and resuspended in 1 mL of growth media (24 g of corn meal, 11 g of soyabean flour, 4 g of NH_4Cl, 15 g of $CaCO_3$, 0.2 g of $MgSO_4$, 50 g of D-glucose, 5 g of soya oil in 1 L of H_2O). One-third of the suspension was used to inoculate 25 mL of sterile media in a 200 mL baffled flask. The culture was incubated in a shaker set at 30 °C, 220 rpm for 4 days. Cells and insoluble media components were spun down, and supernatant was subject to further analysis.

HPLC Fractionation. Samples were brought to 0.1% heptafluorobutyric acid (HFBA) by the addition of 1% HFBA. A Gilson HPLC system consisting of four 306 pumps and a Gilson 215 liquid handler was used to perform the separations. Sample injection volume was 3 mL. Separation was carried out using a 250 × 10 mm Phenomenex Aqua C18 column, with a 50 × 10 mm guard column. Components were eluted using 0.1% HFBA and a gradient of 0−40% acetonitrile (ACN) at a flow rate of 3 mL/min over 45 min. Fractions of 1 mL were collected every 20 s and were assayed without further preparation.

Materials. RNA constructs 16S and 16Sc (Figure 1) were obtained from Dharmacon Research, Boulder, CO. The 16Sc construct contains an 18-atom hexaethylene glycol chain attached to the 5′ terminus of the oligonucleotide as supplied by the manufacturer. The addition of this chain results in a net addition of $C_{12}H_{24}O_9P$ (monoisotopic mass calc 344.1236) to the 16Sc oligonucleotide sequence shown in Figure 1. The RNA was deprotected according to the manufacturer's directions and ethanol precipitated twice from 1 M ammonium acetate. Paromomycin (MW = 615.2963 Da) was obtained from Sigma (St. Louis, MO) and ICN (Costa Mesa, CA).

Mass Spectrometry. Multitarget affinity/specificity screening was performed on a modified Bruker Daltonics (Billerica, MA) Apex II 70e electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer equipped with an actively shielded 7 T superconducting magnet. Experiments were performed with the source at room temperature, the skimmer potential was held at 0 V, the capillary exit potential was −126 V, and other experimental parameters were as described in detail elsewhere.[13] Binding reactions were comprised of 15 μL of a solution containing 2.5 μM 16S and 16Sc in 100 mM NH4OAc and 33% isopropyl alcohol and 2 μL of the LC fraction in a 96-well microtiter plate. Under these conditions, the concentration of HFBA contributed by the HPLC fraction does not interfere with mass spectrometric analysis. The plates were vortexed briefly and then incubated for 60 min at room temperature prior to analysis. Sample aliquots were injected directly from 96-well microtiter plates using a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC). Twenty FTICR scans from each well were coadded, which, along with the overhead associated with the autosampler, resulted in an analysis time of 39 s/well or ∼1 h/96-well plate.

Accurate mass measurements were performed using angiotensin and bradykinin peptides as internal mass standards. These measurements were obtained using a Bruker Apex 9.4 T mass spectrometer. The mass accuracy attained using these standards was ≤1 ppm. Samples were infused at 100 μL/h in 1% formic acid/25% 2-propanol.

Results and Discussion

Disruption of bacterial protein synthesis is the mechanism of action of a number of established antibiotics. Antibiotics such as macrolides and aminoglycosides disrupt protein synthesis by binding to specific sites on one of the bacterial ribosomal subunits. It is known that a number of aminoglycoside antibiotics bind to the *E. coli* 16S rRNA A-site. A synthetic 27-mer RNA corresponding to this site has been shown to bind aminoglycoside antibiotics with the same affinity and specificity as the full length A-site in 16S RNA.[11,15] Two RNA constructs (Figure 1) were used for screening the fractionated bacterial broth. The construct shown on the left (16S) is the 27-mer synthetic RNA containing the *E. coli* 16S ribosomal A-site.[14] Two positions in the *E. coli* 16S RNA are noted numerically as 1408 and 1492, referring to the location of these residues in the intact 16S rRNA. These positions have been previously shown to

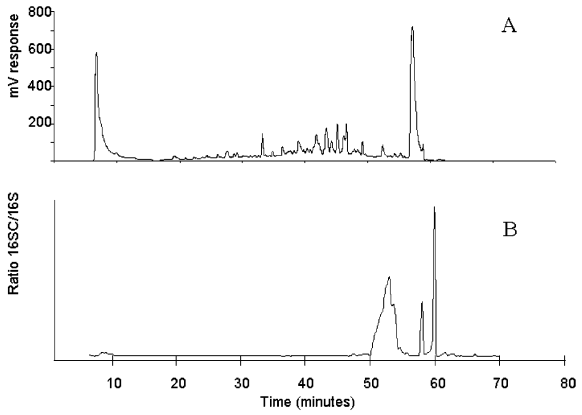

Figure 2. Chromatograms of HPLC reversed-phase fractionation. (A) UV absorption at 254 nm. (B) Plot of the peak intensity ratio of 16SC/16S vs time. The baseline peak intensity ratio is ~0.3 (Figure 1).

be critical for aminoglycoside recognition and binding.[12,15] The second construct, the 16S control construct (16Sc), was created by inserting an additional nucleotide (U) at position 1409, replacing the U at position 1406 with an A, and changing the A's at positions 1408 and 1495 to a G and a C, respectively. A linker was appended to the 5′ terminus of the control sequence to increase the mass difference between the two constructs and minimize the potential for mass ambiguities.[16]

As is typical of ESI-MS spectra of oligonucleotides, low levels of Na^+, K^+, or $NH4^+$ adducts are observed on both constructs (inset, Figure 1). Several low-abundance species are evident between the $(16S)^{-5}$ and $(16Sc)^{-5}$ species (see asterisk in Figure 1) corresponding to an "untagged" synthetic impurity of the 16Sc construct present at ~10.0% relative to the tagged 16Sc construct.

The presence of both a specific (16S) and nonspecific (16Sc) RNA construct enables the simultaneous determination of binding specificity between target and ligand. In the absence of ligand, the initial peak intensity ratio of 16Sc/16S was determined to be 0.3, as shown in the control spectrum shown in Figure 1. The peak intensity ratio of 16Sc/16S should remain constant in the absence of ligand-binding activity. If a ligand specifically binds the 16S RNA target, the peak intensity ratio of 16Sc/16S will increase, while a ligand that binds nonspecifically to both targets will not cause a significant change in the peak intensity ratio of 16Sc/16S. Thus, monitoring the 16Sc/16S peak intensity ratio is a convenient metric for characterizing binding specificity.

The presence of specific and nonspecific RNA-binding species in a bacterial fermentation broth from *S. rimus* sp. *paromomycinus* was determined by multitarget affinity/specificity screening as described in the Experimental Section. Under these growth conditions the broth is expected to contain paromomycin. The UV chromatogram obtained during fractionation of *S. rimus* sp. *Paromomycinus* broth is shown in Figure 2A. The UV trace shows numerous components eluting throughout the 45 min gradient of 0−40% acetonitrile. As a control, a sample of commercially available paromomycin was observed to elute between 52 and 53 min under these conditions (data not shown).

The peak intensity ratio of 16Sc/16S RNA from MASS analysis of all 135 HPLC fractions was plotted as a function of elution time and is shown in Figure 2B. The most significant changes in the peak intensity ratio were observed between ~50 and ~60 min. The rather broad peak between ~50 and ~55 min is consistent with the elution

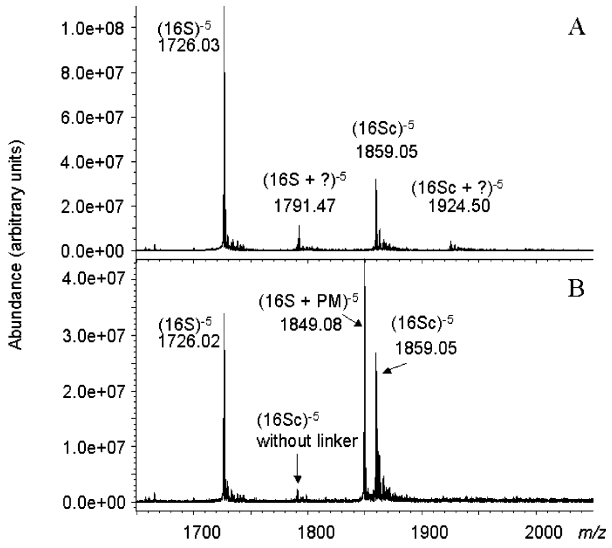

Figure 3. Mass spectra showing paromomycin (PM) binding specifically to 16S RNA (A) and a species with a mass of ~327.25 Da binding nonspecifically to both 16S and 16SC RNA (B). Peak *m/z* values represent the monoisotopic species.

time observed for paromomycin. The high concentration of paromomycin present in the sample resulted in a significantly broader peak compared to the paromomycin standard. Two very sharp peaks were observed at ~58 and ~61 min, corresponding to differential hydrolysis/digestion of the RNA constructs, *vide infra*.

Figure 3A shows the spectrum obtained for fraction 90 (~36 min). In addition to the 5^- charge states of the 16S and 16Sc RNA, two other peaks were observed in the mass spectrum at *m/z* values of 1791.47 and 1924.50 (monoisotopic *m/z* values). These peaks represent noncovalent complexes comprised of an unknown compound and the 16S and 16Sc RNA targets, respectively. The *m/z* difference of 65.45 was observed for the 5^- charge state complex in each case and represents a mass of 327.25 Da for this species. Since the peak intensity ratio of 16Sc/16S did not change appreciably, it can be concluded that this compound does not bind the 16S target with a significant degree of specificity over the 16Sc construct.

The screening results for fraction 131 (~49.5 min) are shown in Figure 3B. In this spectrum, the 5^- charge state of a species at *m/z* 1849.08 is observed, corresponding to a mass difference of 615.30 Da relative to the 16S target. Both the elution time and molecular weight are consistent with paromomycin (monoisotopic mass (calculated) = 615.30). As confirmed below, this peak represents the noncovalent complex between the 16S RNA target and naturally synthesized paromomycin present in the bacterial broth. (The peak broadening, discussed above, results in low levels of paromomycin eluting earlier than the standard and represents the beginning of a peak with a retention time of ~52−53 min.) Importantly, examination of the spectrum in Figure 3B does *not* indicate a peak corresponding to paromomycin binding to the 16Sc RNA (the peak would be expected at an *m/z* of 1982.11), an indication of the specificity of paromomycin for the 16S target RNA over that of the 16Sc RNA. This specificity is also indicated by examination of the peak intensity ratio of 16Sc/16S RNA. In the spectrum shown in Figure 3B, the peak intensity ratio was calculated to be 0.79.

In subsequent fractions, the peak intensity ratio of 16Sc/16S increased as higher concentrations of paromomycin eluted. At sufficiently high paromomycin concentrations the 16S target is completely converted to the 16S−paro-

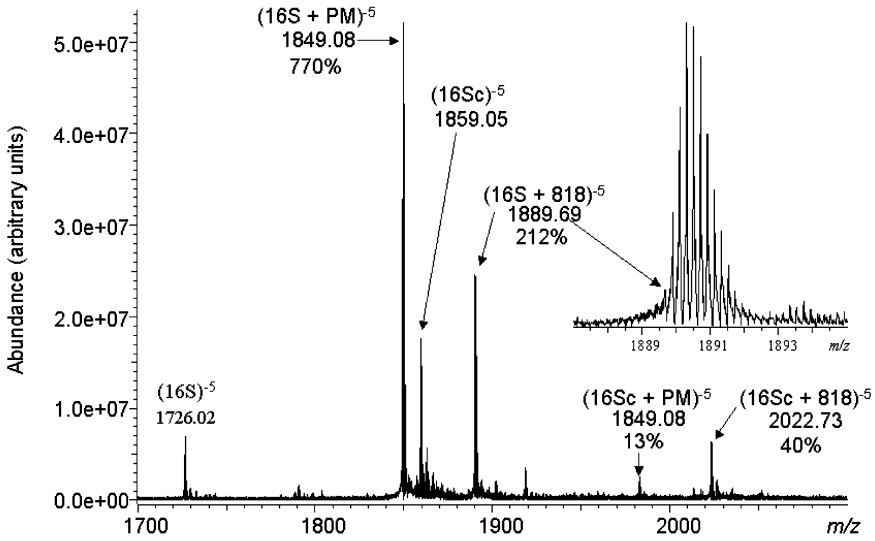

Figure 4. Mass spectrum showing the binding species in fraction 146. The peak *m/z* values represent the monoisotopic species. Two species were observed to bind to 16S RNA: paromomycin (PM) and another species with a molecular weight of 818.35. The percentages shown are relative to the 16S RNA or the 16SC RNA targets, respectively. The inset is a magnification of the noncovalent complex (16S RNA and the 818.35 species) showing the isotope envelope of the 5^- charge state.

momycin noncovalent complex and nonspecific complexes between the 16Sc RNA and paromomycin are also observed, albeit at lower abundance (data not shown). In addition, during the peak of the paromomycin elution (e.g., fraction 138), masses consistent with one to four paromomycin molecules binding to 16S and 16Sc RNA were observed (data not shown).

Under conditions when a very high-affinity ligand is present at a high concentration relative to the target concentration, the 16Sc/16S peak intensity ratio may not be as informative because the binding experiment is being carried out under conditions in which the ligand concentration may be higher than the nonspecific binding constant of the ligand to the 16Sc RNA. In such instances, one can either dilute the fractions that result in complete binding of the target, increase the target concentration, or perform a 2D separation of the fractions prior to re-screening. In any event, it is most prudent to re-screen fractions containing high concentrations of high-affinity ligands, as derivatives and/or isoforms of such ligands may not be chromatographically resolved from the primary binding species. This point is illustrated below with a thorough analysis of fraction 146.

MASS analysis of fraction 146 (~54.7 min) is shown in Figure 4. Noncovalent complexes between the 16S RNA target and two species are apparent at *m/z* 1849.08 and 1889.69. The peak at *m/z* 1849.08 was putatively assigned as the 16S−paromomycin noncovalent complex. The peak at *m/z* 1889.69 represents a different molecule with a mass of 818.35 Da complexed with the 16S RNA target. This new species is also observed at lower abundance as a noncovalent complex with the 16Sc RNA at *m/z* 2022.73. The abundance of the specific complex of 16S + paromomycin compared to 16S was calculated to be 770% (Figure 4), while the abundance of the nonspecific complex of 16Sc + paromomycin was found to be 13%. These results indicate that paromomycin binds approximately 59-fold more specifically to the 16S target than it does to the 16Sc construct. A similar comparison of the new species indicates that it binds with approximately a 5-fold specificity to the 16S RNA target over the 16Sc RNA construct. Similarly, these results indicate that paromomycin binds to the 16S RNA target with approximately 11-fold more specificity than this new molecule (59-fold vs 5-fold).

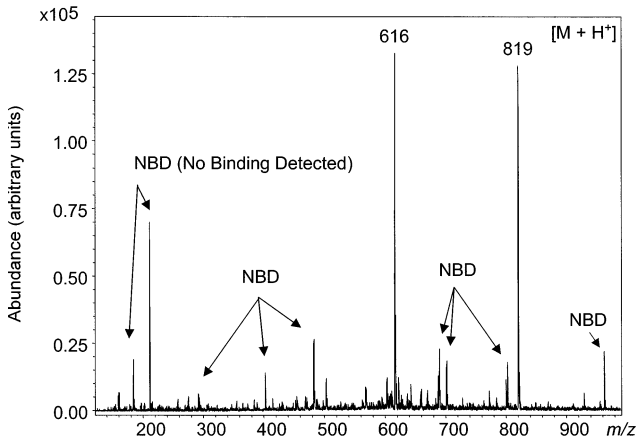

Figure 5. Positive mode mass spectrum showing the species observed in fraction 146.

Figure 5 shows a positive mode mass spectrum of fraction 146. The components observed in fraction 146 include the $(M + H^+)$ species, which is shown below to be paromomycin (*m/z* 616), the $(M + H^+)$ species of the 818 compound (*m/z* 819), and several other species that were present in the fraction, but which did not bind to the 16S or 16Sc RNA (Figure 5). Paromomycin and the unknown compound were the most abundant peaks detected and, assuming that their ionization efficiencies are comparable, were likely present at similar concentrations. Fractions in the vicinity of fraction 146 are particularly interesting, as in addition to containing paromomycin they also contain the 818 species, which based on chromatographic retention and 16S binding, is most likely a paromomycin derivative.

An accurate mass measurement of the presumed paromomycin ($(M + H^+) \approx 616$) from fraction 146 was performed on a 9.4 T FTICR mass spectrometer. Mass accuracy with sub-ppm mass measurement error was achieved using internal mass standards. The mass was measured to be 616.3035 ± 0.0006 ($C_{23}H_{46}O_{14}N_5$ calc 616.3036). MS/MS fragmentation of this species gave daughter ions consistent with those of paromomycin[17−20] (data not shown).

The MS/MS spectrum produced from isolation and fragmentation of the novel species generated a daughter ion at *m/z* 616. Further fragmentation of this daughter ion

resulted in daughter ions consistent with those of paromomycin[17−19] (data not shown). These data suggest that the 819 species is composed of a core paromomycin moiety that has been modified on one or more of its rings. Work is in progress to further characterize this novel binding compound.

Fractions 156 (~58 min) through 162 (~60 min) demonstrated relatively sharp peaks in the peak intensity ratio plot of 16Sc/16S (Figure 2). A number of peaks were observed with masses from ~650 to ~7700 Da, which were not evident in the direct ESI-MS analysis of the fractions. These masses were compared with potential degradation products of the 16S and 16Sc targets and were found to match these expected products (data not shown) and correspond to RNA oligonucleotides in the 2-mer to 24-mer size range. These data suggest that the constituents of these fractions induced limited hydrolysis of both targets. The 16S RNA appeared to be hydrolyzed to a greater degree than the 16Sc RNA when the levels of each were compared, possible due to the neutral mass tag "cap" on the 5′ end of the 16Sc construct or the tighter stem structure of the fully Watson−Crick base-paired stem.

These data suggest that, in addition to finding small molecules that bind to biomolecular targets of interest, MASS can be used to identify natural product fractions with functional activity consistent with proteases, transferases, reductases, kinases, etc. Further studies are presently underway to explore this approach with both RNA and protein targets.

Conclusions

In this work, we demonstrate high-throughput mass spectrometric detection of known and unknown species that bind to relevant RNA targets directly from crude natural product fractions. Examples of compounds binding nonspecifically and specifically were shown. Furthermore, the ability to concomitantly compare the binding of a given molecule to a specific RNA target and a control RNA target was shown to be a convenient way to track the specificity of potential drug candidates. The knowledge gained in tracking molecules that bind both specifically and nonspecifically is important in the development of structure−activity studies using traditional medicinal chemistry.

In the search for new drug candidates from natural products, the use of MASS to determine the binding properties of compounds present and to provide accurate mass measurements, coupled with chromatographic response and tandem MS, can identify unknowns and go a long way toward full structural elucidation of novel ligands. Compound identification and the structural information obtained are equally important in subsequent medicinal chemistry studies. This information would most likely be missed using traditional biological activity assays or would be more difficult to obtain, as larger quantities of natural product would have to be processed in order to obtain sufficient quantities of material to test.

The ability to use MASS to screen RNA-binding components in natural products clearly opens the door to further investigations on bacterial broths. The observation of fractions that bind to and hydrolyze the RNA target clearly demands further study, especially if the activity in the fractions is nonproteinaceous. Other exciting areas for future study include examination of natural product broths derived from various growth conditions and stages, screening cell lysates for bioactive compounds that are not secreted, and determining the RNA-binding characteristics of biosynthetic products from organisms containing gene-shuffled synthetic pathways.

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